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Citation for published version:

van 't Hof, R & Ralston, SH 1997, 'Cytokine-induced nitric oxide inhibits bone resorption by inducing apoptosis of osteoclast progenitors and suppressing osteoclast activity' *Journal of Bone and Mineral Research*, vol. 12, no. 11, pp. 1797-804. DOI: 10.1359/jbmr.1997.12.11.1797

Digital Object Identifier (DOI):

[10.1359/jbmr.1997.12.11.1797](https://doi.org/10.1359/jbmr.1997.12.11.1797)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Journal of Bone and Mineral Research

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Cytokine-Induced Nitric Oxide Inhibits Bone Resorption by Inducing Apoptosis of Osteoclast Progenitors and Suppressing Osteoclast Activity

ROBERT J. VAN 'T HOF and STUART H. RALSTON

ABSTRACT

Interferon- γ (IFN- γ) has been shown to inhibit interleukin-1 (IL-1) and tumor necrosis factor α (TNF- α) stimulated bone resorption by strongly stimulating nitric oxide (NO) synthesis. Here we studied the mechanisms underlying this inhibition. Osteoclasts were generated in 10-day cocultures of mouse osteoblasts and bone marrow cells and the effect of cytokine-induced NO on osteoclast formation and activity was determined. Stimulation of the cocultures with IL-1 β , TNF- α and IFN- γ markedly enhanced NO production by 50- to 70-fold, and this was found to be derived predominantly from the osteoblast cell layer. When high levels of NO were induced by cytokines during early stages of the coculture, osteoclast formation was virtually abolished and bone resorption markedly inhibited. Cytokine stimulation during the latter stages of coculture also resulted in inhibition of bone resorption, but here the effects were mainly due to an inhibitory effect on osteoclast activity. At all stages, however, the inhibitory effects of cytokines on osteoclast formation and activity were blocked by the NO-synthase inhibitor L-NMMA. Further investigations suggested that the NO-mediated inhibition of osteoclast formation was due in part to apoptosis of osteoclast progenitors. Cytokine stimulation during the early stage of the culture caused a large increase in apoptosis of bone marrow cells, and these effects were blocked by L-NMMA and enhanced by NO donors. We found no evidence of apoptosis in osteoblasts exposed to high levels of cytokine-induced NO at any stage in the culture, however, or of apoptosis affecting mature osteoclasts exposed to high levels of NO, suggesting that immature cells in the bone marrow compartment are most sensitive to NO-induced apoptosis. In summary, these studies identify NO as a potentially important osteoblast-osteoclast coupling factor which has potent inhibitory effects on bone resorption. These actions, in turn, are mediated by inhibition of osteoclast formation probably due to NO-induced apoptosis of osteoclast progenitors and by inhibition of the resorptive activity of mature osteoclasts. (J Bone Miner Res 1997;12:1797-1804)

INTRODUCTION

NITRIC OXIDE (NO) is a free radical that has been shown to play an important role in the regulation of vascular tone, platelet function, neurotransmission, and immune function.⁽¹⁾ An action of NO that is the source of increasing interest is as a mediator of bone cell function.⁽²⁾ Proinflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor (TNF), and interferon gamma (IFN- γ) have potent effects on bone remodeling⁽³⁾ and are also powerful stimulators of NO production in bone cells,⁽⁴⁻⁶⁾ raising the

possibility that NO may act as a mediator of cytokine effects on bone turnover. In keeping with this hypothesis, NO has been found to be an important modulator of osteoclastic bone resorption. At low concentrations, NO acts to potentiate IL-1-induced bone resorption,⁽⁷⁾ and evidence has been presented to suggest that constitutive production of small amounts of NO may be necessary for normal osteoclast function.⁽⁸⁾ High concentrations of NO strongly inhibit bone resorption, however, both in organ cultures^(7,9) and in cultures of isolated osteoclasts.^(8,10,11) While high levels of NO have been shown to be responsible for the inhibitory

effect of IFN- γ on IL-1 and TNF- α stimulated bone resorption,^(7,9) the mechanisms by which NO exerts its effects on the osteoclast remain unclear.

To define these mechanisms, we studied the effects of cytokine-induced NO production in cocultures of mouse calvarial osteoblasts and bone marrow cells.⁽¹²⁾ In this system, fully differentiated and actively resorbing osteoclasts form over a 10-day culture period, thus permitting a detailed analysis of the relative effects of NO on osteoclast formation and activity.

MATERIALS AND METHODS

Isolation and culture of cells

The bone marrow–osteoblast coculture system used is an adaptation of that described by Takahashi et al.⁽¹²⁾ Calvarial bones were dissected from 2-day-old Balb/C mice and treated for 15 minutes with 1 mg/ml collagenase type I (Sigma Chemical Co., St. Louis, MO, U.S.A.) in Hank's balanced salt solution (HBSS). The supernatant was discarded and the calvariae were digested with collagenase for another 30 minutes. The resulting cell suspension was removed, and the cells were resuspended in culture medium (α -MEM, supplemented with 10% fetal calf serum [FCS] and penicillin/streptomycin). The calvariae were washed in phosphate buffered saline (PBS) and treated for 15 minutes with 4 mM EDTA in PBS followed by a third 30-minute collagenase digestion. Both of the resulting cell suspensions were spun down and resuspended in culture medium, and all three cell suspensions were pooled. The cells were allowed to adhere to a 75 cm² tissue culture flask, nonadherent cells were removed, and the remaining cells cultured overnight in culture medium at 37°C and 5% CO₂, rinsed with PBS and harvested by trypsin digestion. The resulting cell population consisted predominantly of osteoblasts; >90% of the cells stained positive for alkaline phosphatase, less than 0.1% of the cells stained positive for the macrophage marker acid phosphatase, and none of the cells stained positive for the endothelial marker von Willebrand Factor.

A bone marrow cell population containing osteoclast precursors was isolated from the long bones of 3- to 5-month-old Balb/C mice, by flushing marrow from dissected long bones with a syringe and a 25 gauge needle using HBSS supplemented with 10% FCS. Erythrocytes were removed by Ficoll Hypaque density gradient centrifugation, and the remaining bone marrow cells were washed with PBS, spun down, and resuspended in culture medium.

Cocultures of osteoblasts and bone marrow cells were performed in either 48- or 96-well tissue culture plates. In 96-well plates, the osteoblasts and bone marrow cells were plated at 10⁴ cells/well and 2 \times 10⁵ cells/well, respectively, in 150 μ l of α -MEM supplemented with 10% FCS, antibiotics, and 10 nM 1,25-dihydroxyvitamin D₃. In 48-well plates, double the amount of cells and culture medium were used. At the end of the culture period, osteoclasts were identified by tartrate resistant acid phosphatase (TRAP) staining and resorption pits by reflected light microscopy (see below). In some experiments, calvarial osteoblasts

were replaced by UMR 106 osteoblast-like cells, plated at the same density.

In the coculture system, formation of osteoclasts can be divided in three different stages. During the first 3 days, no TRAP positive mono- or multinucleated cells are observed. Subsequently (days 4–7), TRAcP positive cells start to appear and the first resorption pits are usually detected at day 5 or 6, and later still (days 7–10), large numbers of multinucleated TRAcP positive multinucleated cells (MNCs) are present and extensive resorption takes place.

Tartrate resistant acid phosphatase staining

Cultures were fixed for 10 minutes in buffered formalin (pH 7.4), washed with PBS, and incubated with naphthol-ASBI-phosphate, hexazotized pararosanilin, and 30 mM tartrate in acetate buffer (pH 5.5) at 37°C for 30 minutes.⁽¹³⁾ The cultures were washed with PBS and the number of osteoclasts, defined as TRAP positive stained multinucleated (>3 nuclei/cell) cells, were counted.

Analysis of resorption pits

Osteoblasts and bone marrow cells were cocultured on top of dentine slices (elephant tusk, 4 mm diameter) in 96-well plates as described above. At the end of the culture period, the cells were removed from the dentine slices and the resorption pits examined directly by reflected light microscopy. The area of resorption was measured using a Leitz Q500MC image analysis system (Leitz, Milton Keynes, U.K.).

Nitric oxide production

NO production was assessed by measuring the stable end product of NO, nitrite, in the culture medium using the Griess reaction as described previously.⁽⁴⁾

Detection of apoptosis

Apoptotic cells were identified by typical appearances on light and electron microscopy and by the TUNEL (TdT-mediated X-dUTP nick end labeling) assay, using a kit obtained from Appligene Oncor (Chester-Le-Street, U.K.). For the TUNEL assay, cocultures were performed as described above in tissue culture chambers, and at the end of the culture period the cells were fixed in 4% paraformaldehyde. The cultures were then incubated with terminal deoxy/nucleotidyl transferase and digoxigenin (DIG)-labeled nucleotides according to the manufacturer's instructions. Apoptotic DIG-labeled nuclei were identified using a fluorescein isothiocyanate (FITC)-labeled anti-DIG antibody. In some studies, apoptosis was also quantitated using a commercially available kit, which measures DNA fragmentation by an enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim, Lewes, U.K.). Osteoblasts and bone marrow cells were incubated overnight with the thymidine analog 5-bromo-2'-deoxy-uridine (BrdU). Subsequently, the cells were cocultured for varying periods and all the cells lysed with a nonionic detergent. The ELISA

(which detects BrdU-labeled DNA fragments) was performed according to the manufacturer's instructions, and apoptosis (in arbitrary units) was expressed as absorbance at 540 nm using 690 nm as the reference wavelength. BrdU labeling efficiency of the osteoblast and bone marrow cell populations was similar (>95%) as determined by immunocytochemical labeling (data not shown).

Statistics

Student's *t*-test was used in statistical evaluation, and differences were scored as significant when the two-tailed *p* value was less than 0.05.

RESULTS

Cytokines stimulate NO production in cocultures

Stimulation of the cultures with cytokines led to a moderate induction of NO synthesis by IL-1 β and TNF- α and strong stimulation by IFN- γ (Fig. 1a). There was significantly greater stimulation of NO production with combinations of two cytokines, and the highest amounts were produced upon stimulation of the cells with all three cytokines (cocktail). Irrespective of the stimulus, NO synthesis was significantly inhibited by the competitive nitric oxide synthase (NOS) inhibitor L-N^G-monomethyl-L-arginine (L-NMMA) (Fig. 1b). When osteoblasts and bone marrow cells were cultured separately and exposed to the cytokine cocktail, both could be stimulated to produce NO, although the amount of NO (measured as medium nitrite) produced per cell was more than 100-fold greater in the osteoblasts (118 pmol/cell/h) than in the bone marrow cells (0.83 pmol/cell/h). This was reflected by the fact that the osteoblast monocultures produced much more NO than bone marrow monocultures and almost as much NO as the cocultures (Fig. 1b), even though the bone marrow monocultures and cocultures contained approximately 20 times more cells than the osteoblast cultures. Taken together, these data suggest that, in the coculture system, cytokine-induced production of NO is predominantly derived from osteoblast-like cells. Basal and cytokine-stimulated NO production did not differ significantly in parallel cultures which were performed on plastic as opposed to those cultured on dentine (compare for example, NO₂ levels in Figs. 1a and 1b [plastic] with those in Fig. 2c [dentine]).

Effects of cytokine-induced NO on osteoclast formation and activity

When the cytokine cocktail was added during the early stages of osteoclast development (days 4–7), hardly any osteoclasts formed (Fig. 2a) and pit formation (measured at day 10, Fig. 2b) was almost completely inhibited. Both effects were partially reversed when NO production was blocked by the NOS inhibitor L-NMMA, indicating that NO was largely responsible for the effects observed. When the cultures were cytokine stimulated at a later stage in the culture, by which time many osteoclasts had already formed (days 7–10), a small increase in osteoclast number was

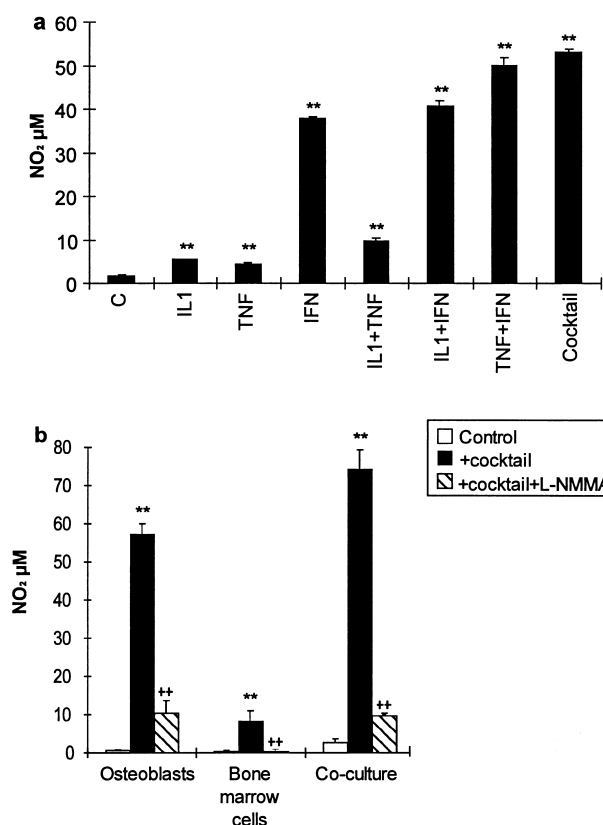


FIG. 1. Cytokine-stimulated NO production by cocultures is mainly derived from osteoblasts. Osteoblasts and bone marrow cells were isolated and cultured for 2 days as described in the Materials and Methods section, cytokines were then added and medium nitrite levels measured after a further 48 h. (a) Cocultures of osteoblasts and bone marrow cells stimulated with cytokines as indicated (IL-1, 10 U/ml; TNF, 25 ng/ml; IFN, 100 U/ml). (b) Osteoblast cultures, bone marrow cultures, and cocultures were set up separately and stimulated with cytokine mix (IL-1, 10 U/ml; +TNF, 25 ng/ml; +IFN, 100 U/ml) in the presence and absence of the NOS-inhibitor L-NMMA (1 mM). The results shown are from a representative experiment from at least three performed, with three replicates per group. **p* < 0.05, ***p* < 0.01 from control, ++*p* < 0.01 from no L-NMMA.

observed compared with controls (Fig. 2a) but there was a significant decrease in resorption area (Fig. 2b), indicating less resorption per osteoclast. Simultaneous addition of L-NMMA at this stage led to a further increase in the number of osteoclasts and a marked increase of bone resorption. Taken together, these results indicate that cytokine-induced NO production exerts a powerful inhibitory effect on osteoclast formation at all stages of the culture but demonstrates that when NO is induced during the later stages of culture, there is an additional inhibitory effect of NO on resorptive activity of mature (preformed) osteoclasts. The differential effects on osteoclast formation and activity did not appear to be due to major differences

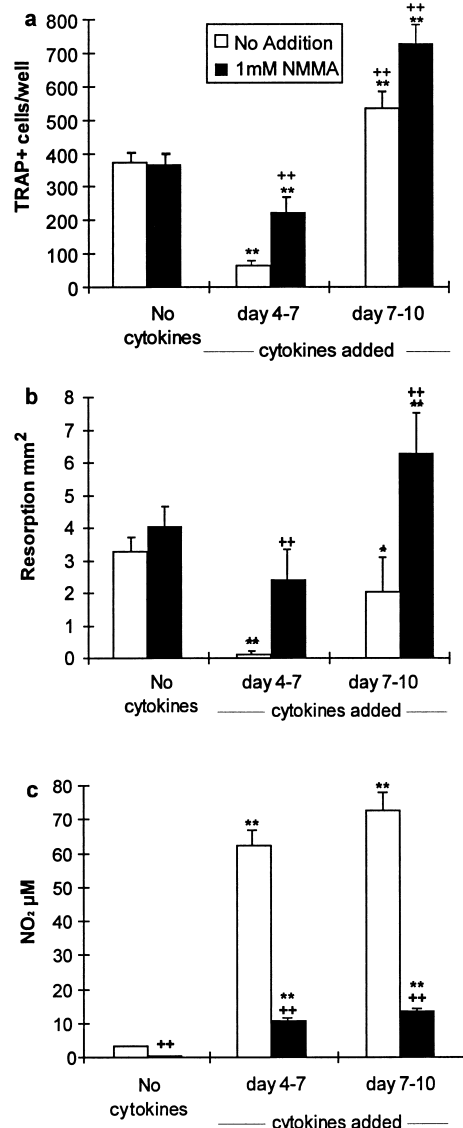


FIG. 2. Effect of cytokine-induced NO on bone resorption at different stages of osteoclast development. Cocultures were performed on dentine slices (b, c) or in 48-well tissue culture plates (a). The cytokine mix (IL-1, 10 U/ml; TNF, 25 ng/ml; IFN, 100 U/ml) with or without 1 mM L-NMMA, was added at day 4 or day 7. Medium in cultures that were cytokine stimulated on day 4 was replaced with fresh medium on day 7 which did not contain cytokines, and NOS activity was inhibited with 1 mM L-NMMA for the remaining 3 days (days 7–10). In all cultures, resorption area and osteoclast numbers (number of TRAP positive cells) were measured on termination of the experiments at day 10. The results shown are from a representative experiment from at least three performed, with five replicates per group. * $p < 0.05$, ** $p < 0.01$ from no cytokines, + + $p < 0.01$ from no L-NMMA.

between the degree of NO stimulation at different stages in the culture since NO₂ levels were only marginally higher in cultures stimulated with cytokines between days 7–10 as compared with those stimulate between days 4–7 (Fig. 2c).

NO induces apoptosis of bone marrow cells in cocultures

Large numbers of cell fragments were observed in the nonadherent (bone marrow-derived) cell layer in cocultures that had been stimulated with the cytokine cocktail, whereas these effects were not observed in control cultures (Fig. 3). Since cellular fragmentation is a feature of apoptosis, we decided to investigate the possibility that cytokine-induced NO production was inhibiting osteoclast formation by causing apoptosis of osteoclast progenitors. Large numbers of apoptotic cells were identified by TUNEL staining in the nonadherent bone marrow cell layer, and the presence of apoptosis was confirmed by electron microscopy (Fig. 4), which demonstrated typical features, including nuclear fragmentation, and nuclear and cellular condensation. A large increase in apoptosis as measured by a DNA fragmentation ELISA was also observed in the nonadherent cell layer after cytokine stimulation and this was reversed by the addition of L-NMMA (Fig. 5). It is important to emphasize that cytokine-induced apoptosis was only observed in cocultures and not in single cultures of either osteoblasts or bone marrow cells (Fig. 5) which suggests that the high levels of NO produced by osteoblasts in the cocultures are necessary for apoptosis to occur. Indeed, the low basal level of apoptosis, which was present in bone marrow cells cultured in the absence of osteoblasts, was significantly decreased by treatment with the cytokine cocktail. Cytokine-induced apoptosis was noted to a similar extent in cocultures performed on plastic and on dentine slices (data not shown).

To investigate the possibility that factors specific to the coculture system such as osteoblast–bone marrow contact may have contributed to the apoptotic effects of cytokines, we performed further studies in which murine bone marrow cells were cocultured with UMR 106 osteoblast-like cells in place of calvarial osteoblasts, since our previous studies had shown that UMR 106 cells do not produce significant amounts of NO in response to cytokine stimulation (Ralston, unpublished data). Monocultures of UMR 106 cells produced low levels of NO₂ (mean \pm SEM = $0.5 \pm 0.25 \mu$ M), and this was not significantly increased by the cytokine cocktail ($0.75 \pm 0.30 \mu$ M; $p = \text{NS}$). Furthermore, cocultures of UMR 106 cells and murine bone marrow cells produced only marginally more NO than bone marrow cells alone in response to stimulation with the cytokine cocktail ($10 \pm 3 \mu$ M vs. $8 \pm 2 \mu$ M; $p = \text{NS}$) and in these cultures, the cytokine cocktail did not stimulate apoptosis (data not shown).

The importance of NO, rather than cytokines, as the mediator of apoptosis was further emphasized by experiments in which apoptosis was measured in response to stimulation with individual cytokines and combinations of two or more cytokines in the presence and absence of L-NMMA. Here we found a close correlation ($r = 0.885$; $p < 0.01$) between the number of apoptotic cells and the concentrations of NO achieved after cytokine stimulation (Fig. 6). When the cocultures were stimulated with single cytokines, only IFN- γ stimulated apoptosis. The combination of IL-1 and TNF- α did not induce apoptosis, whereas

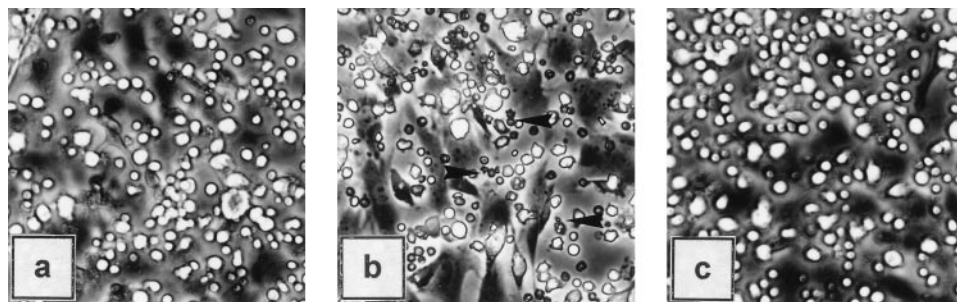


FIG. 3. Cytokine-stimulated NO production causes apoptosis of bone marrow cells in cocultures. Osteoblasts and bone marrow cells were cocultured for 3 days, stimulated with a cytokine mix (IL-1, 10 U/ml; TNF, 25 ng/ml; IFN, 100 U/ml) and the experiment terminated after a further 48 h. (a) Phase contrast photomicrograph of unstimulated culture, showing healthy bone marrow cells. The osteoblast layer below (slightly out of focus) remains intact. (b) Parallel culture stimulated with the cytokine mix. Compared with the control culture, the stimulated culture contains a large number of shrunken cells and cell fragments (arrowheads). The underlying osteoblast layer remains intact. (c) Parallel culture stimulated with the cytokine cocktail in the presence of 1 mM L-NMMA, showing reversal of the effects of the cytokine mix on cell morphology by L-NMMA. Again, the underlying osteoblast layer remains intact. The results shown are from a representative experiment from at least three performed, with three replicates per group.

the combination of IFN- γ with IL-1 resulted in a moderate induction of apoptosis, and the combination of TNF- α and IFN- γ resulted in apoptosis levels comparable to those in cocultures treated with the full cytokine cocktail. Experiments performed in the presence of the NO inhibitor L-NMMA showed low levels of apoptosis, in keeping with the data presented in Figs. 3–5.

To determine whether high concentrations of NO alone were sufficient to induce apoptosis, we treated the cocultures with the pharmacological NO donors S-nitroso-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP). While neither agent was sufficient to induce apoptosis on its own in concentrations of up to 500 μ M (equivalent to medium NO₂ levels of >100 μ M), both NO donors reversed the inhibitory effects of L-NMMA on apoptosis in cocultures stimulated with the cytokine cocktail (representative experiment with SNAP; Fig. 7).

These data strongly suggest that it is cytokine-induced NO, rather than the cytokines themselves, or other osteoblast-released factors that mediate apoptosis of bone marrow cells in the coculture system. While these experiments confirm that NO is essential for cytokine-induced apoptosis of osteoclast precursors, they also show that NO donors alone are insufficient to induce apoptosis, at least at concentrations which deliver NO at levels equivalent to those resulting from cytokine stimulation. This suggests that the proapoptotic effects of NO in this system may be enhanced in the context of a cytokine induced costimulus.

Bone marrow cells are the main target for NO-induced apoptosis

To confirm that bone marrow-derived cells were indeed the main target for apoptosis, we performed cocultures where only one of the cell populations was prelabeled with BrdU and measured DNA fragmentation (reflecting apoptosis) using an ELISA (Fig. 8). When only the osteoblasts were prelabeled with BrdU, no increase in DNA fragmen-

tation was detected after cytokine stimulation, whereas a large increase was observed when the bone marrow cells were labeled, almost identical to that when both populations were labeled. This indicates that the vast majority of apoptotic cells in the coculture are derived from the bone marrow population. Labeling of apoptotic cells using the TUNEL method also showed that immature cells in the bone marrow population were the main target for apoptosis. In cocultures that were exposed to cytokines at an early stage (days 2–4), a high percentage of the nonadherent bone marrow cells were labeled (mean \pm SEM = $63 \pm 3.7\%$, compared with $6 \pm 1\%$; $p < 0.007$) in the unstimulated control cultures. In contrast, very few osteoblasts (<1%) were labeled by TUNEL staining after cytokine stimulation.

Although the presence of apoptotic cells in the nonadherent cell population does not completely exclude the possibility that some of these cells may have derived from the osteoblast layer, this seems extremely unlikely, taking into account the results of the selective BrdU labeling experiments (Fig. 8), the absence of apoptosis in osteoblast monocultures (see above), and the fact that the large increase in apoptotic cells observed in the cocultures (63% of 2×10^5 cells) far exceeds the total number of osteoblasts present in the cultures.

It was also of interest that very few labeled mature osteoclasts were seen in cocultures stimulated with cytokines during the later stages (days 7–10), suggesting that immature cells present in the bone marrow cell population are the primary target for NO-induced apoptosis in this system, rather than mature, fully differentiated osteoclasts.

DISCUSSION

This study shows that both osteoclast formation and the activity of mature osteoclasts is inhibited by high levels of cytokine-induced NO and that the decreased osteoclast formation, in turn, is almost certainly due to NO-induced

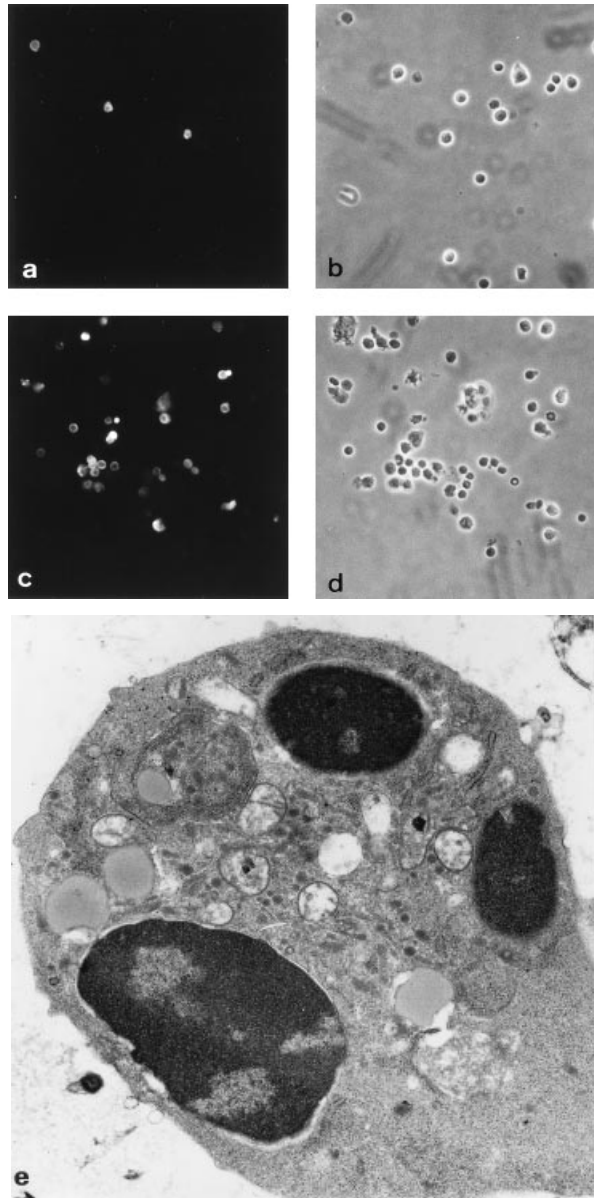


FIG. 4. Confirmation of NO-mediated cytokine-induced apoptosis in bone marrow cells by TUNEL staining and electron microscopy. Osteoblasts and bone marrow cells were cocultured for 3 days and subsequently stimulated with a cytokine mix (IL-1, 10 U/ml; TNF, 25 ng/ml; IFN, 100 U/ml). Nonadherent cells were harvested 24 h after stimulation by aspiration of the wells and stained using the TUNEL method (a) and (c) or analyzed by transmission electron microscopy (e). (a) Control cultures, TUNEL stain; (b) phase contrast image of (a); (c) parallel cytokine stimulated culture, TUNEL stained, showing an increase in labeled stained cells; (d) phase contrast image of (c); (e) electron micrograph of cytokine stimulated culture, showing fragmented and condensed nucleus with loss of normal chromatin structure, condensed cytoplasm and intact organelles and cell membrane. The results shown are from a representative experiment from at least three performed, with three replicates per group. Overall, the percentage of TUNEL labeled apoptotic cells was much greater in cytokine-stimulated cultures (c) than in control cultures (mean \pm SEM = $63 \pm 7\%$ vs. $6 \pm 1\%$, $p < 0.007$).

apoptosis of osteoclast progenitors. These data agree with previous experiments which have shown that high levels of NO inhibit bone resorption in organ cultures of bone^(7,9) and in cultures of mature osteoclasts.^(8,10,11) The absence of apoptosis in previous studies of bone organ cultures and in cultures of mature osteoclasts exposed to high concentrations of NO induced by cytokines⁽⁷⁻¹¹⁾ supports our data which show that immature cells in the bone marrow population are the main target for NO-induced apoptosis, rather than mature osteoclasts or osteoblasts. This is in keeping with the observation that highly proliferative cells, like osteoclast progenitors, are much more susceptible to apoptosis than differentiated cells^(14,15) like mature osteoclasts. The failure of L-NMMA to completely restore bone resorption and osteoclast formation to control levels was probably attributable to the fact that NO production could not be completely inhibited, even by high concentrations of L-NMMA, especially when cytokines were added early during the culture period. While inhibition of bone resorption also occurred when high levels of NO were induced during the later stages of culture, this effect appeared to be due, in part, to inhibition of resorption by mature osteoclasts, as manifest by the marked reduction in pit formation, in the presence of a significant increase in osteoclast numbers. We found no evidence, either on morphological grounds or on TUNEL staining, to suggest that high levels of cytokine-induced NO caused mature osteoclasts to undergo apoptosis.

Although it has been suggested that constitutive production of NO may be important in regulating osteoclast activity,^(8,11) we found no effect of the NOS inhibitor L-NMMA on osteoclast numbers or pit formation in unstimulated cultures. From this we can conclude that in this system at

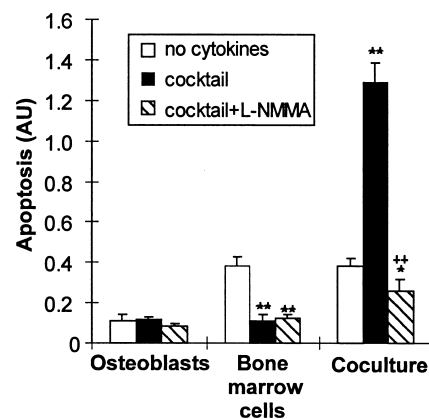


FIG. 5. Cytokine-induced apoptosis only occurs in cocultures. Osteoblast cultures, bone marrow cultures, and cocultures were stimulated on day 3 with a cytokine mix (cocktail) of IL-1 (10 U/ml) + TNF (25 ng/ml) + IFN (100 U/ml) in the presence and absence of L-NMMA (1 mM). Apoptosis was quantitated 48 h after cytokine stimulation, using a DNA fragmentation ELISA (see Methods). The results shown are from a representative experiment from at least three performed, with three replicates per group. * $p < 0.05$, ** $p < 0.01$ from no cytokines, + $p < 0.01$ from no L-NMMA; $n = 3$.

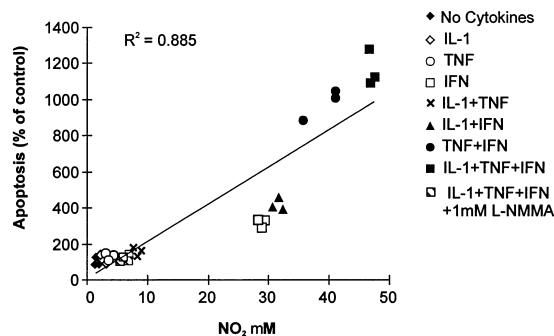


FIG. 6. Correlation between cytokine-induced NO levels and apoptosis in cocultures. BrdU labeled osteoblasts and bone marrow cells were cocultured for 3 days and stimulated with cytokines (IL-1, 10 U/ml; TNF, 25 ng/ml; IFN, 100 U/ml), alone and in combination as indicated in the presence or absence of L-NMMA. Apoptosis was quantitated 48 h after cytokine stimulation, using the DNA fragmentation ELISA as described in the text. The line in the graph represents the calculated linear regression line of the combined experiments each of which was run with three to five replicates per treatment group.

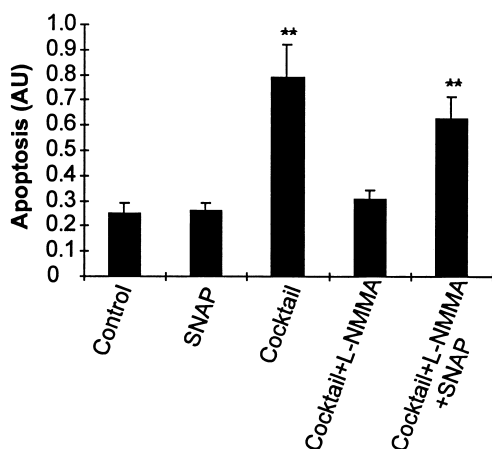


FIG. 7. NO is essential but not sufficient to induce apoptosis in cocultures. BrdU-labeled osteoblasts and bone marrow cells were cocultured for 3 days and stimulated with a cytokine mix (cocktail) of IL-1 (10 U/ml) + TNF (25 ng/ml) + IFN (100 U/ml) alone and in combination with SNAP (300 μ M) and/or L-NMMA (1 mM). Apoptosis was quantitated using a DNA fragmentation ELISA as described in the text. The results shown are from a representative experiment from at least three performed, with four replicates per group. ** $p < 0.01$ from control, SNAP and cocktail/L-NMMA groups.

least, constitutive production of NO plays a limited role in regulating osteoclast formation and activity.

It is of interest that early osteoclast progenitor cells present within the bone marrow cell fraction appeared to be an important target for the proapoptotic effects of cytokine-induced NO. Apoptosis was not increased in the osteoblast layer on exposure to high concentrations of cytokine-induced NO, and apoptosis in single cultures of bone marrow

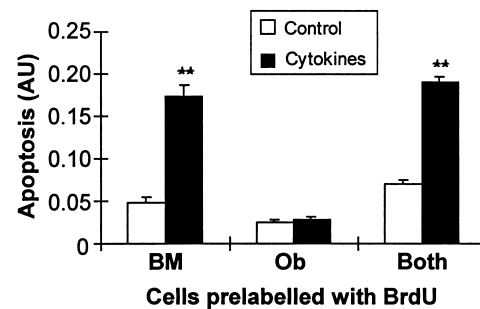


FIG. 8. Bone marrow cells are the main target for NO-induced apoptosis. Cocultures were set up in which the cell populations were individually labeled with BrdU and then cytokine stimulated with a mix of IL-1 (10 U/ml) + TNF (25 ng/ml) + IFN (100 U/ml). BM, bone marrow cells BrdU labeled; Ob, osteoblasts BrdU labeled; Both, bone marrow cells and osteoblasts BrdU labeled. Apoptosis was quantitated 24 h after cytokine stimulation by a DNA fragmentation ELISA as described in the text. Cytokine-induced increases in DNA fragmentation were detected by the ELISA only when bone marrow cells were prelabeled with BrdU and the amount of fragmentation was identical to that obtained when both cell populations were BrdU labeled, indicating that cytokine-induced DNA fragmentation (reflecting apoptosis) predominantly occurs in the bone marrow population. The results shown are from a representative experiment from at least three performed, with four replicates per group. ** $p < 0.01$ from control cultures.

cells was inhibited upon exposure to the cytokine mix. In agreement with our data, high levels of IFN- γ and TNF- α induced NO have been shown to inhibit proliferation of human bone marrow cells,⁽¹⁶⁾ and this inhibitory effect has been partially attributed to NO-induced apoptosis.⁽¹⁷⁾ The fact that cytokines reduced apoptosis in bone marrow cell monocultures may be attributed to a positive effect of cytokines on bone marrow cell survival, coupled with the fact that monocultures of bone marrow cells produced only a small amount of NO after cytokine stimulation.

Osteoblasts appeared to be the main producers of NO in this system, since the rate of NO production in monocultures of osteoblasts was over 100 times greater than that of bone marrow cell cultures and almost as great as in the cocultures. Although it is possible that bone marrow cells could have acquired the ability to produce NO at a later stage in the culture period as they differentiated into more mature cells, the contribution of bone marrow cell-derived NO is likely to be small, since the total levels of NO produced in response to cytokines did not increase substantially with time during the later stages of the culture.

The fact that osteoblasts acted as the main source of NO is relevant to previous studies, which have shown that many osteotropic factors do not act directly on osteoclasts, but rather act indirectly by inducing production of substances by osteoblasts which then influence osteoclast formation and activity.⁽¹⁸⁾ The osteoblast-produced substances are poorly defined, but, like NO, are thought to be small, diffusible molecules which act over short distances between

cells which are in close contact. The coculture system used here now shows that cytokine-induced NO released from osteoblasts can act directly in a relatively selective fashion to down-regulate osteoclast formation and activity, thereby identifying NO as a potentially important osteoblast-osteoclast coupling factor.

The importance of NO as a mediator of cytokine-induced apoptosis of osteoclast precursors is supported by the observation that the effect could be largely reversed by inhibition of NOS with L-NMMA. However, experiments using the NO donors SNAP and SNP showed that these agents alone cannot induce apoptosis, at least at concentrations that deliver NO at equivalent levels to those which result from cytokine stimulation. This indicates that while high levels of NO are necessary to trigger apoptosis in this situation, they are not sufficient to do so and must be delivered in the context of a cytokine costimulus. This in turn implies that NO may interact with cytokine-induced signaling pathways in bone to induce apoptosis, although the nature of these interactions will require further research.

In summary, high levels of osteoblast-produced NO inhibit bone resorption by two mechanisms: first, by inducing apoptosis of osteoclast progenitors present in the bone marrow cell population, and second, by inhibiting the resorptive activity of mature osteoclasts. The first mechanism accounts for the potent inhibitory effect of cytokines on osteoclast formation during the early stages of the coculture, whereas the second mechanism is important during the later stages where we observed increased osteoclast numbers, but decreased resorption, indicating less resorption per osteoclast. The former mechanism is in keeping with previous observations which have shown an absence of osteoclasts in bone organ cultures treated with IL-1, TNF, and IFN, and the latter with the observation that NO donors inhibit resorption by mature isolated osteoclasts.^(10,11)

These studies confirm that NO is a potent inhibitor of bone resorption with effects on both osteoclast formation and activity. Pharmacological modulation of NO may therefore represent a new approach in the treatment of bone diseases characterized by increased bone resorption, such as osteoporosis, rheumatoid arthritis, and cancer-associated osteolysis.

ACKNOWLEDGMENTS

We thank the Arthritis and Rheumatism Council of the U.K. and the Chief Scientist's Office of the Scottish Home & Health Department for grant support.

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Received in original form March 6, 1997; in revised form May 22, 1997; accepted June 10, 1997.